Moderate alcohol exposure during early brain development increases stimulus-response habits in adulthood

Matthew O. Parker[a]

Alexandra M-D. Evans[a]

Alistair J. Brock[a]

Fraser J. Combe[a,c]

Muy-Teck Teh[b]

Caroline H. Brennan[a]*

[a]*School of Biological and Chemical Sciences, Queen Mary University of London, London E1 4NS*

[b]*Centre for Clinical and Diagnostic Oral Sciences, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, E1 2AD*

[c]Present address: *Manchester Metropolitan University, Manchester, M15 6BH*

*Correspondence relating to this article should be addressed to Caroline H. Brennan. Email: c.h.brennan@qmul.ac.uk

Abstract

Exposure to alcohol during early central nervous system (CNS) development has been shown variously to affect aspects of physiological and behavioural development. In extreme cases, this can extend to craniofacial defects, severe developmental delay and mental retardation. At more moderate levels, subtle differences in brain morphology and behaviour have been observed. One clear effect of developmental alcohol exposure is an increase in the propensity to develop alcoholism and other addictions. The mechanisms by which this occurs, however, are not currently understood. In this study we tested the hypothesis that adult zebrafish chronically exposed to moderate levels of ethanol during early brain ontogenesis would show an increase in conditioned place preference (CPP) for alcohol and an increased propensity towards habit formation, a key component of drug addiction in humans. We found support for both of these hypotheses and found that the exposed fish had changes in mRNA expression patterns for dopamine receptor, nicotinic acetylcholine receptor (nAChR) and μ-opioid receptor (MOR) encoding genes. **Collectively, these data show an explicit link between the increased proclivity for addiction and addiction-related behaviour following exposure to ethanol during early brain development, and alterations in the neural circuits underlying habit learning.**

Keywords: CPP; moderate prenatal ethanol; habit; zebrafish; addiction; μ-opioid; dopamine

Introduction

Maternal alcohol consumption during pregnancy results in a range of effects on the developing foetus, collectively referred to as Foetal Alcohol Spectrum Disorders (FASD; Paintner et al., 2012). FASD are characterized by a range of teratogenic and psychological defects, and represent the leading non-hereditary cause of mental retardation, with the prevalence estimated at between 2 and 5% of the population of the USA and western Europe (May et al., 2009). In extreme circumstances, when mothers drink heavily during pregnancy, foetal alcohol syndrome (FAS) results in gross skeletal and craniofacial abnormalities, and severe CNS dysfunction (Hanson et al., 1976). More moderate alcohol consumption (e.g., equivalent to 1-2 drinks/day, average BAC ~0.01-0.04g/dL Valenzuela et al., 2012), however, is associated with a range of subtle cognitive and behavioural defects, including aggression and affective disorders (Sood et al., 2001), impulsivity and inattention (Streissguth et al., 1989; Suess et al., 1997), poor scholastic performance (Olson et al., 1998) and deficits in social behaviour (Keil et al., 2010; Kelly et al., 2000; Thomas et al., 1998).

Exposure to moderate ethanol during gestation also poses an increased risk that the offspring will develop substance abuse problems in later life (Valenzuela et al., 2012). For example, Baer et al. (2003) demonstrated in a 21-year prospective study that adults who had been exposed to ethanol during gestation had a higher incidence of alcohol abuse. This study, and others like it (Alati et al., 2006; Alati et al., 2008; Miller, 1999; Streissguth et al., 1994) have typically controlled very robustly for the effects of post-natal extraneous environmental mediators (e.g., parental role models, etc.), and there is also evidence from adoption studies that suggest direct cause-effect relationships

(Landgren et al., 2010; van Wieringen et al., 2010). Nevertheless, clinical studies have only limited efficacy in describing the cellular and molecular mechanisms by which ethanol exposure during gestation exerts its effects on addiction in later life.

Preclinical studies using animal models have proved very useful in this regard for a number of reasons. First, it is possible to titrate alcohol intake in a precise manner. Second, it is possible to exclude potential confounds (e.g., post-natal environment, poly-drug abuse, etc.). Third, the gestational period during which alcohol is present can be carefully scrutinised. Fourth, the offspring can be monitored and tested at various developmental stages. For example, extensive work with rodent models have variously demonstrated that ethanol during early brain development affects key components of the mesoaccumbens dopamine reward pathway, potentially explaining why affected individuals show greater drug preference in later life (Blanchard et al., 1993; Fulginiti et al., 1994; Middaugh et al., 1994; Wang et al., 2006).

Although these studies have been helpful in determining the impact of developmental ethanol exposure on structural and functional alterations in midbrain dopamine systems, simply increasing the reward potential of a drug is not sufficient to explain the development of addiction to that (or other) drugs (Everitt and Robbins, 2005; Robbins and Everitt, 1999). A key aspect of addiction is the progressive ‘loss of control’ over drug use and the establishment of learned habitual (compulsive) drug taking. As midbrain dopaminergic circuits are involved in the establishment of habit learning (Balleine and Dickinson, 1998), this raises the possibility that developmental ethanol exposure leads to an alteration in the learning processes such that there is an increased propensity to develop habits, and thus an increased likelihood of developing addiction.

During instrumental learning, responses are controlled by two distinct processes. The first, response-outcome (R-O) learning, is seen early in the learning process and is flexible in the sense that it is affected by alterations in contingencies and by devaluation of the reinforcer. The second process, stimulus-response (S-R), or 'habit' learning is far less flexible, is not affected by alterations in contingencies or devaluation, and is often seen as a result of over-training (Balleine and Dickinson, 1998). This form of behavioural plasticity has not only been characterized in a variety of vertebrates (rodents; (Balleine and Dickinson, 1998), primates; (Fernandez-Ruiz et al., 2001), horses; (Parker et al., 2009)), but also in invertebrates (drosophila; (Brembs, 2009)). The shift between R-O and S-R learning is adaptive: in a changing environment, the cost associated with learning about potentially important new information must be balanced with the ability to learn rules relating to continuously performed tasks. For example, it would make driving a car a far more arduous task if we had to consult a manual before every trip. In mammals these processes, collectively known as action selection, are directly mediated by dopamine signalling between the prefrontal cortex (PFC) and the dorsal striatum (DS). Specifically, the medial aspect of the DS (DMS) is essential for R-O learning and the lateral aspect (DLS) for S-R learning (Balleine and Dickinson, 1998).

Moderate prenatal ethanol exposure causes alterations in dendritic morphology in the shell region of the nucleus accumbens (NAcs) in rats (Rice et al., 2012b). The nucleus accumbens (NAc) is functionally and structurally separated into the NAcs and the core region (NAcc). NAcs is primarily thought to be involved with the processing of the incentive value of reward, whereas the NAcc is controls instrumental learning (Corbit et al., 2001). Reductions in spine

density and dendritic length in the NAcs following prenatal ethanol exposure in rats (Rice et al., 2012b) suggests that the increase in preference for ethanol is not the result of a generalized increase in the incentive value of rewards. Instead, it may be mediated by an augmented role for the NAcc in instrumental learning increasing DS control of behaviour. In turn, this could lead to accelerated shift from DMS-controlled R-O learning to DLS-controlled S-R habit (Yin et al., 2008). We therefore designed this study to test the hypothesis that exposure to moderate amounts of ethanol during early brain development would increase the development of S-R habits in adulthood, using zebrafish as a model species.

Over recent years, the zebrafish has emerged as one of the major vertebrate species in behavioural neuroscience, with procedures emerging showing clear translational relevance to human disorders such as drug addiction (Guo, 2004; Kalueff et al., 2013; Parker et al., 2013a). This has included conditioned place preference for drugs of abuse (CPP; Brennan et al., 2011; Darland and Dowling, 2001; Kily et al., 2008; Ninkovic et al., 2006), impulsivity (Parker et al., 2014; Parker et al., 2013c) as well as compulsive drug seeking and relapse (Brennan et al., 2011; Kily et al., 2008). **One of the key benefits of using zebrafish to complement mammalian models is their small size and genetic tractability. In addition, their transparency as embryos facilitates exploration of developmental effects through *in vivo* imaging. This, coupled with the fact that a number of brain systems linked to addiction in mammals are well conserved in zebrafish, or at the very least, functional homologues have been identified, makes them a potentially useful model. For example, pallial structures such as the amygdala, hippocampus and piriform cortex are conserved, and the ascending midbrain dopamine pathways (in**

mammals, the ventral tegmental area [VTA] to NAc to prefrontal cortex [PFC]) is present in teleost fish such as zebrafish (in zebrafish, the VTA [posterior tuberal nucleus; PTN] and NAc [ventral [Vv] and dorsal [Vd] telencephalic nuclei] (see Parker et al., 2013a for recent reviews; Parker and Brennan, 2012). In addition, we have recently introduced zebrafish as a model for moderate prenatal ethanol exposure, showing that adults exposed to low-moderate levels of ethanol during early brain development have differences in social cohesion and social exploratory behaviour and that these differences may be mediated by differences in 5-HT circuits (Parker et al., In Press).

Previous research in adult zebrafish found brain concentrations of ethanol following chronic exposure typically reach ~80-90% bath concentration in adults (Dlugos and Rabin, 2003; Mathur et al., 2011) and ~30-40% in embryos and larvae (Reimers et al., 2004). The relationship between brain and blood alcohol content is not straightforward, and estimates for the ratio of brain:blood ethanol range from 0.6-1.5 (Moore et al., 1997). Therefore, we exposed embryos to 20mM ethanol which would result in BAC of ~0.02-0.07g/dL, putting the zebrafish model in the moderate prenatal ethanol classification (Valenzuela et al., 2012). The aims of this study were to test the hypotheses that moderate developmental ethanol exposure would increase both drug preference and the formation of S-R habits**.** In addition, we aimed to characterize mRNA expression in a variety of genes that encode proteins related to addiction and habit formation, including the dopamine receptors [DR] (Robbins and Everitt, 1999), dopamine transporter (DAT) (Hironaka et al., 2004), nicotinic acetylcholine receptors [nAChRs] (Kitabatake et al., 2003), μ-opioid receptor [MOR] (Miura et al., 2008), adenosine *a2a* receptor

(Yu et al., 2009), and noradrenaline transporter [NAT] (Xu et al., 2000). **We chose these genes specifically as they have previously been shown to affect aspects of habit formation and compulsive drug seeking. Many other genes may also be involved with these behaviours and this is not meant as an exhaustive list.**

Materials and Methods

Subjects

Adult Tubingen (mixed male/female) zebrafish were kept in a re-circulating system, on a 14/10-hour light/dark cycle, at ~28.5°C within the zebrafish aquarium facility at Queen Mary University of London. **There have been mammalian studies that have distinguished between male and females. However, as the genetic work was carried out on larvae, we would not be able to assign a sex to the individuals at this age. Therefore, all animals were randomly assigned to the treatment conditions**. Fish were fed with a mixture of flake food, fresh brine shrimp and bloodworm. Adults were bred in house and fry reared according to standard protocols (Westerfield, 1993). Larvae from each condition (20mM ethanol and control) were sacrificed at 24hpf (i.e., before ethanol exposure), 50hpf (acute ethanol exposure: 2hrs after ethanol added), 72hpf and finally at 7dpf. All animal work was carried out following approval from the Queen Mary Research Ethics Committee, and under licence from the Animals (Scientific Procedures) Act 1986. Care was taken to minimize the numbers of animals used in this experiment in accordance with the ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357). Specifically, we examined data from pilot studies in our lab, our previous published work (Parker et al., In Press) and studies with other species (Rice et al., 2012b) to carry out a power calculation and assess the minimum number of animals necessary for the expected effect size with power of 0.8.

Developmental ethanol exposure

The developmental ethanol exposure is described in detail elsewhere (Parker et al., In Press). Tubingen zebrafish larvae were treated by transferring them into 20mM (0.12% v/v, equating to ~0.04g/dL BAC) ethanol in aquarium water at 48 hpf. Prior to ethanol treatment, all larvae were staged to ensure they were at the correct developmental stage (Kimmel et al., 1995). In addition, any larvae that were still in their chorions were dechorionated to ensure equal exposure to ethanol. We chose 48hpf to start treating the larvae as it represents a key stage in CNS development, the Long Pec stage (Guo et al., 1999; Kimmel et al., 1995). Furthermore, we quantified the size of larvae to ensure that there were no differences between the treatment groups that might affect gene expression analysis using a commercially available automated visualisation system (www.elaborant.com). Previous research in adult zebrafish found brain concentrations of ethanol following chronic exposure typically reach ~80-90% bath concentration in adults (Dlugos and Rabin, 2003; Mathur et al., 2011) and ~30-40% in embryos and larvae (Reimers et al., 2004). The relationship between brain and blood alcohol content is not straightforward, and estimates for the ratio of brain:blood ethanol range from 0.6-1.5 (Moore et al., 1997). Therefore, we estimate that the larvae would have had a blood alcohol concentration (BAC) of ~0.02-0.07g/dL putting the zebrafish model in the moderate prenatal ethanol classification (Valenzuela et al., 2012).

Fish water was changed on alternate days, and the tanks were cleaned, in order to reduce the build-up of yeast and control variation in the ethanol concentration due to evaporation. Larvae were kept in Petri dishes until they were five days old, after which they were transferred into tanks with dimensions 10 x 10 x 20 cm (depth x width x length cm) and a volume of 500ml with airlines. A

maximum of 50 fish were kept in each tank and numbers of ethanol and control fish were balanced; dividing equally the quantity of fish receiving ethanol or aquarium water for the controls between the tanks. Feeding of the larvae ZM000 and paramecium commenced at five days. After seven days swimming in fish water containing ethanol (i.e., aged 9 days), the fry were transferred back to pure aquarium water. At age three weeks the volume of water in the tanks was increased to 1L to provide more space for growth and the ZM000 was replaced with ZM100 and artemia. At age five weeks they were transferred into 7L tanks and cleaning was reduced to once a week. Control fish were treated identically, except that the ethanol was not added to their water at any time.

Conditioned place preference

The conditioned place preference procedure comprised an opaque tank measuring (W x L x H) 16.5cm x 33cm x 15 cm and containing 3L of water (see Parker et al., 2013b for detailed methods). The visual disciminanda (black spots or black vertical lines) were located on the walls of the tanks (see supplementary Figure 1). Conditioning started on a Monday, when the fish were habituated. All fish were first placed individually in the conditioning tanks, with no ethanol added, for a period of 20-mins. This was repeated on the second day (Tuesday), during which time baseline preference for spots or stripes was recorded by measuring time spent in each area of the tank. On the following two days (Wednesday-Thursday), the fish were conditioned to their least-preferred stimulus, as determined by their baseline preference. During conditioning trials, all fish were initially exposed to the non-drug side for a period of 20 minutes. An opaque

barrier in the tank prevented access to the drug side. After 20-minutes, the barrier was lifted to allow the fish to move to the drug-paired side of the tank. On entry, the barrier was replaced and ethanol was added to the tank water. **In order to avoid a concentration gradient through the tank, we carefully poured half of the ethanol solution into opposite corners at the far ends the tank (see supplementary Figure 1)**. **We added pure ethanol mixed with distilled H_2O to the tanks to avoid 100% ethanol being poured directly onto the fish, which may have been aversive. In other words, 1.5 ml of ethanol was added to 48.5ml aquarium water, and this solution was added to the test tank.** This procedure was repeated on the following 2 days. Following conditioning, all fish were tested in a single probe trial, during which they were placed in a tank with the barrier lifted such that both sides could be accessed freely. Time spent in the proximity to the drug-paired stimulus was recorded. **This procedure was used initially to determine the appropriate dose of ethanol. To do this, we tested control fish at 0, 0.5, 1, and 1.5% ethanol. As a result 0.5% ethanol was chosen as a threshold dose to use when comparing responses of developmentally treated and control fish.**

Habit formation

In order to test habit formation, we used a fish-version of the widely used Tolman T-maze assay (supplementary Figure 2). This is the first time this has been used in fish, but has been used in mammals regularly to measure aspects of habit formation and S-R learning (Packard and Knowlton, 2002; Yin and Knowlton, 2004; Yin et al., 2004). Fish were initially habituated to the apparatus for two half-hour sessions prior to testing. During training sessions, the

fish was initially placed in the test area in the South arm (nb, the release arm and choice arms were counterbalanced between fish). The barrier was then removed to allow the fish access to the choice arms. The barrier was closed behind the fish. If the fish chose the East arm, a barrier was placed behind it and it was reinforced with a small amount of brine shrimp (correct response). The barrier remained in place for 20-seconds to ensure the fish had time to consume the food. If the fish entered the West arm, it was given 60 seconds to choose the correct arm, after which it was guided into the correct arm and fed. This ensured that each trial was reinforced to remove any potential differences in reinforcement history between the fish. Each fish was trained for 5 trials at a time (one training block = 5 trials) until they reached criterion (< 20-sec mean approach latency for 5 consecutive sessions and mean correct responses ≥75%). Once the fish had reached criterion, they were tested in a single probe trial. This was always carried out in the fifth trial of a 5-trial block. In other words, trials 1-4 were normal training trials, and trial 5 was the probe. During the probe trial everything was exactly the same as during training trials except that the fish was released from the opposite arm to that from which it was released in training. During the probe trial, no reinforcement was given. The experimenter noted which arm the fish entered on release from the starting arm. The experimenter was blind to treatment allocation at all times during testing to eliminate any bias (fish were allocated a number, and their group identity not revealed before data analysis).

qPCR procedures

Larvae at 24hpf, 50hpf, 72hpf and 7dpf in batches of n = 3 embryos were homogenized in 200µl Lysis buffer with 2µl Proteinase K for 30-45min (55ºC).

mRNA was isolated using 80µl Dynabeads® Oligo($dT)_{25}$ according to manufacturer's instructions. Reference genes (see Table 1 for primer sequences) were chosen according to previous research *β-actin*, *ef1α* and *rpl13α* (Parker et al., In Press; Tang et al., 2007). Target genes used were nicotinic acetylcholine receptors (nAChRs; *chrna2-7, chrnb2-4*), dopamine receptors and transporter (DAT; *drd1-3, slc6a3*), noradrenaline receptor (NAT; *slc6a2*) and µ-opioid receptor (MOR; *oprm1*). Absolute quantification was obtained by making standards for each gene, prepared using the relevant primers to amplify fragments from cDNA. Samples were then PCR purified and diluted to 10^{11} fragments using the Avogadro constant. All qPCR reactions were carried out in triplicate. 2µl of cDNA and 2µl each of forward and reverse primers (see Table 1) was added to 10µl SYBR® Green PCR Master mix (Applied Biosystems) on a LightCycler LC480 instrument (Roche Diagnostic). For detailed methods see (Gemenetzidis et al., 2010; Teh et al., 2013).

[TABLE 1 HERE]

Data analysis

Statistical analyses were carried out in IBM SPSS for Macintosh (version 21). All null hypotheses were evaluated with respect to $\alpha = 0.05$. Normality, homoscedacity and homogeneity of variance were ascertained by examination of fitted vs. residual plots from the models. **With respect to the CPP dose-response curve, data were fitted to a quadratic regression (dependent variable = change in preference for the drug-predictive cue from baseline, independent variable = dose of ethanol (0, 0.5, 1, 1.5% v/v).** Data pertaining to the comparison of CPP between ethanol-treated and control fish was analysed using a 2-way mixed design analysis of variance (ANOVA), with one between-

subjects factor (group: ethanol-exposed vs. handling control), one within-subjects factor (phase: pre- vs. post-conditioning), as well as their interaction (group x phase). The dependent variable was time spent in proximity to the conditioned cue.

With respect to T-maze acquisition, the effects of developmental ethanol treatment on latency to approach the target arm, and proportion of correct responses, were fitted by restricted maximum likelihood (REML) to linear mixed effects model (LME). Denominator degrees of freedom were estimated using the Satterthwaite approximation (Satterthwaite, 1946). Latency data were fitted to a model with Gaussian error structure by REML, and correct responses were fitted to a logistic regression model (binomial distribution) by Laplace approximation. For the binomial model, significance was ascertained from Type-III Wald Chi-Squared statistics performed on the final model. Training block (1-31), group (ethanol-exposed vs. handling control) and their interaction, were added to initial models as fixed effects. Fish-ID was added to both models as a random effect to control for associated interclass correlation (random-intercept model). Finally, probe trial data (frequency of 'habit' vs. 'place' responses for ethanol-exposed or handling control fish) was analysed using a Fisher's exact test. qPCR data were analysed with 2-way factorial ANOVAs. The between-subjects factors were age (4-levels: 24hpf, 48hpf, 72hpf, 7dpf) and treatment (2-levels: ethanol vs handling control). Descriptive statistics are reported as mean ± SE.

Results

Moderate ethanol exposure during early brain development does not affect unconditioned locomotor behaviour

First, we examined the basal locomotor responses of the adult fish that had been exposed as embryos to 20mM ethanol. This information is extremely relevant, since ethanol may impair motor coordination depending on both time of exposure and concentration. Without this, it may be difficult to comprehend how such data can be related to memory, since behavioural tasks in animal models is directly dependent of their locomotor profile. We placed the fish individually (n = 14 treated with 20mM ethanol as embryos, n = 14 control) in a circular container (radius = 30cm) and filmed them for 10 min (Noldus Ethovision, TrackSys, Nottingham UK). We measured the total distance travelled over the 10 mins (cm) as well as the number of rotations (i.e., complete body rotations about the central axis of the body) to examine any gross motor effects of the ethanol. Independent t-tests revealed no effects of ethanol on distance (t [26] = 1.09, P = 0.29; control = 9427 ± 654.02 cm 20mM ethanol = 8475.38 ± 581.04 cm) or rotations (t [26] = 0.39, P = 0.7; control = 16.29 ± 4.2 rotations, 20mM ethanol = 19.43 ± 6.97 rotations).

Moderate developmental ethanol exposure increases CPP for ethanol

[FIGURE 1 ABOUT HERE]

Figure 1A displays the dose-response curve to ethanol. There was a significant quadratic effect on change in preference as a function of dose, $F(2, 42) = 7.89$, $P = 0.001$, $R^2 = 0.27$ (regression parameter estimates: $y = -0.04+0.41*x^2-0.2*x$). The highest point on the peak was at 1%, but to avoid potential ceiling effects in the developmental ethanol experiment, we used 0.5% v/v to condition our fish.

Figure 1B displays the time spent in the vicinity of the conditioned cue (i.e., spots or stripes, depending on least preferred during basal preference assessment) before (basal) and after (probe trial) conditioning with 0.5% ethanol. Fish that were developmentally exposed to 20mM ethanol showed a greater increase in preference for the drug-paired cue following conditioning. These patterns were confirmed with an LME. There was a main effect of phase, $F(1,75) = 16.28$, $p < 0.001$, but not group, $F < 1$. There was also a significant group x phase interaction, $F(1,75) = 5.3$, $p = 0.02$, characterized as the ethanol exposed group showing a greater increase in preference for the conditioned cue during the probe trial ($p < 0.01$). In summary, developmental exposure to 20mM ethanol significantly augmented the increase in preference for the drug-paired cue following CPP training with 0.5% v/v ethanol.

Moderate developmental ethanol exposure increases motor habit formation

[FIGURE 2 ABOUT HERE]

Figure 2 displays the acquisition data for fish during training on the T-maze assay. Performance was variable throughout in both groups, but ethanol treated fish showed marginally poorer learning during the training overall. In terms of latency to respond (Figure 2A), an LME revealed no main effect of group, $F < 1$,

but there was a main effect of block, F (30, 1788) = 2.43, $p < 0.0001$ and a significant group x block interaction, F (30, 1788) = 1.79, $p < 0.01$. The interaction was characterized as the ethanol fish showing longer latencies in blocks 3, 18, 21 and 22 (ps < 0.05).

In terms of correct responses during each session, we fitted a logistic regression model by Laplace approximation. There was a significant main effect of block (Figure 2B), χ^2 (30) = 103.26, $p < 0.0001$, with correct responses increasing after block 21. There was a marginally non-significant main effect of group, χ^2 (1) = 3.37, $p = 0.06$, and there no significant group x block interaction, χ^2 (30) = 33.9, $p = 0.28$. To summarise, all fish appeared to have learnt the discrimination, although there was some evidence from latency data that the fish exposed developmentally to 20mM ethanol learnt at a slightly slower rate than handling controls.

During the probe trial, 75% of the fish exposed to ethanol appeared to show a motor habit, turning in the direction they had turned during training. Of the fish from the handling control group, 100% went to the arm reinforced during training in the probe trial, suggesting that none of them had adopted a habit. This difference was confirmed with a Fisher's exact test (Figure 2C; $p = 0.01$).

Moderate developmental ethanol exposure affects mRNA expression of nicotinic, dopaminergic and μ-opioid receptors

[FIGURE 3 ABOUT HERE]

There was an effect of ethanol treatment on *chrna2* mRNA expression that approached significance, F (1,24) = 3.96, $P = 0.058$, and a significant effect of

age, F (3,24) = 6.4, $P < 0.01$. There was also a significant age x treatment interaction (see Figure 3A), F (3,24) = 3.92, $P < 0.05$, characterized as expression being significantly supressed in ethanol-treated 50hpf embryos [$P < 0.05$]. There was a significant effect of age on *chrna3* mRNA expression, F (3,24) = 5.38, $P < 0.01$, but no significant effect of prenatal ethanol treatment, F (1,24) = 2.12, $P = 0.16$. There was, however, a significant age x treatment interaction (see Figure 3B), F (3,24) = 2.95, $P = 0.05$, characterized as expression being supressed at 50hpf in the ethanol-treated embryos [$P < 0.05$]. There was a significant effect of age on *chrnb3* expression, F (3,24) = 14.68, $P < 0.01$, but no effect of treatment, F (1,24) = 1.57, $P = 0.22$. There was also a significant age x treatment interaction (see Figure 3C), F (3,24) = 7.45, $P = 0.001$, characterized as ethanol causing an increase in mRNA expression at 50hpf [$P < 0.01$], followed by a reduction in expression at 7dpf [$P < 0.05$].

There was a significant effect of age on *slc6a3* mRNA expression, F (3, 24) = 5.67, $P < 0.01$, but no effect of treatment, F (1,24) = 3.67, $P = 0.07$. There was a significant age x treatment interaction (see Figure 3D), F (3,24) = 3.45, $P = 0.03$ characterized as there being an increase in mRNA expression at 50hpf, but only in the control group (i.e., expression was supressed by ethanol treatment) [$P = 0.03$]. There was a significant effect of age on *drd3* mRNA expression, F (3,24) = 5.55, $P < 0.01$, but no effect of treatment, $F < 1$. There was a significant age x treatment interaction (see Figure 3E), F (3,24) = 3.27, $P = 0.04$ characterized by expression being significantly increased in the ethanol-treated group at 50hpf [$P < 0.01$], but this effect reversed at 7dpf [$P < 0.05$]. There was a significant effect of age on *oprm1* mRNA expression, F (3,24) = 4.55, $P = 0.012$, but no effect of treatment, $F < 1$. There was also a significant age x treatment interaction (Figure

3F), F (3,24) = 3.26, $P < 0.05$, characterized as ethanol significantly increasing mRNA expression at 50hpf [$P < 0.05$] but this effect reversed at 7dpf [$P < 0.05$].

There were no significant effects of ethanol treatment on mRNA expression for the other nAChR-encoding genes (*chrna4, chrna5, chrna6, chrna7, chrnb2, chrnb4*), nor those encoding for the other dopamine receptors (*drd1, drd2*), adenosine receptor (*adr2a, adr2b*) or noradrenaline transporter (*slc6a2*), nor were there any age x treatment interactions (see **Supplementary Results**).

Discussion

Exposure to moderate levels of ethanol during early brain ontogeny increases preference for alcohol and other drugs in the offspring, and acts as a significant risk factor for the development of addiction. The aim of this experiment was to explore the mechanism by which this manifests by examining behavioural and gene expression changes following exposure to moderate levels of ethanol during brain development in zebrafish. Exposure to 20mM (0.12% v/v, equivalent to 0.2-0.7 g/dL BAC) ethanol from 48hpf-9dpf increased the time spent in the presence of the ethanol-conditioned cue in the CPP test as compared to non-exposed controls, suggesting an increased preference for alcohol in the exposed fish as has been previously observed in rodents. **We also found that after training on a place-response test in a modified Tolman T-maze, there was a difference in performance at the specific time point at which the criterion as selected were met, suggesting that exposed fish were more likely to show S-R learning early in the learning process, whereas non-exposed controls**

showed R-O learning. This indicated an increased propensity for habit formation in developmentally exposed individuals. Finally, we found that ethanol acutely supressed *chrna2, chrna3* and *slc6a3* mRNA expression. It also acutely increased *chrnb3, drd3* and *oprm1* mRNA expression, but this effect reversed by 7dpf for all three suggesting ethanol caused adaptation in expression of these genes during early brain development. Our data support the hypothesis that animals developmentally exposed to ethanol show increased S-R learning and suggests involvement of dopaminergic, opioid and nicotinic pathways in this process.

We found that ethanol increases preference for alcohol in a CPP test, in agreement with previous work showing that prenatal ethanol exposure increases alcohol preference in later life in humans (Spear and Molina, 2005) and in rodents (Arias and Chotro, 2005; Van Waes et al., 2011). Importantly, this shows the translational relevance of fish in this procedure. Two main theories to explain increased self-administration seen following developmental exposure to ethanol exist in the literature. The first is that ethanol preference is caused by the chemosensory adaptation of the foetus developing in an alcoholic medium (Dominguez et al., 1993; Faas et al., 2000). This theory has been challenged in particular by the observation that exposed offspring show a higher preference for other rewarding drugs as well as ethanol (Barbier et al., 2009). An alternative theory is that ethanol alters structural and functional neuroplasticity, particularly in the striatum and other midbrain structures, and this alters reward circuitry. There is some evidence for changes in neural pathways associated with reward following developmental ethanol (Blanchard et al., 1993; Choong and Shen, 2004; Malanga and Kosofsky, 1999; Middaugh et al., 1994). However, an

increased preference for alcohol (or other drugs) does not necessarily translate in to an increased propensity for addiction (Everitt et al., 2008). Indeed, preclinical studies have found that there is no clear correlation between self-administration rates and the development of compulsive drug seeking or relapse, suggesting that other processes are more important (Belin et al., 2008).

In addition to an increased preference for ethanol, we found that fish exposed to ethanol during early brain development appeared to shift from R-O to S-R earlier in the learning process. Although they learned the task marginally slower than the controls, in a probe trial where the fish were released from the opposite side of the maze, the ethanol fish all showed a motor habit, whereas the majority of the controls used the extra-maze cues to guide navigation. This difference in stimulus control relatively early in the learning process suggests that the ethanol exposed animals were displaying less goal-directed, cognitive control over their behaviour, and more automated 'habitual' responses, an effect typical of those addicted to drugs (Balleine and Dickinson, 1998). Thus, our findings suggest that exposure to ethanol during early brain development enhances habit formation, potentially rendering the exposed animal more susceptible to addiction. This is particularly interesting in the light of the previously discussed brain changes observed following developmental ethanol exposure in rodents, where rats exposed to moderate ethanol during gestation had a decrease in MSN spine density and dendritic length in the NAcs (Rice et al., 2012b). As these regions are key to the process of instrumental contingency learning, this provides a mechanism potentially linking developmental ethanol to enhanced habit formation (i.e., ventral to dorsal shift; Belin et al., 2013), fitting with current theories that characterise addiction in terms of maladaptive habits where drug seeking comes

under the control of conditioned reinforcers (drug-related discriminative stimuli) (e.g., Everitt and Robbins, 2005; Everitt and Robbins, 2013).

As developmental ethanol exposure has been shown to alter gene expression in a number of pathways, we examined expression of components of pathways associated with the switch from R-O to S-R learning (e.g., dopamine, opioid and adenosine) as well as other systems associated with drug seeking and addiction (adrenergic and cholinergic) in larval fish. We found that ethanol caused a number of changes in mRNA expression in genes relevant to addiction. First, we found that ethanol initially increased and then decreased, *drd3* expression, the gene that encodes for the DRD3. This supports previous work with rats that showed prenatal ethanol exposure reduced quiniprole (a DRD2/3 agonist)-induced yawning (Brus et al., 1995). Lower levels of DRD2/3 in the ventral striatum have been linked both to poor inhibitory control and to escalation of self-administration rates for cocaine in rodents (Dalley et al., 2007). In addition, DRD3 expression has been linked specifically to habit formation, with schizophrenia patients and healthy controls with a variant (Ser9Gly polymorphism) at the DRD3 locus showing stronger habit learning (Kéri et al., 2005). We did not see any differences for DRD1 or DRD2 expression, or in the adenosine a2a receptor (which forms a heterodimer with DRD2; Franco et al., 2000), but we did see an acute effect on DAT with ethanol-exposed embryos showing suppression of *slc6a3* mRNA expression at 50hpf (2hrs after ethanol was added to the embryo medium), with expression normalising thereafter. DAT-KO mice show resistance to extinction, suggesting increases in habit-like learning (Hironaka et al., 2004). Here, there were no differences in expression at 7dpf, but we did not test the fish in adulthood for DAT. The fact that *slc6a3* mRNA

552 expression was acutely suppressed by ethanol (i.e., at 50hpf) suggests that there
553 may be some long-term effects on general DAT function, and this could be
554 studied in more detail in future experiments.

555 In addition to finding what appeared to be an adaptive change in DRD3,
556 we observed an acute increase in *orpm1* mRNA expression (50hpf) followed by a
557 decrease in expression at 7dpf. Again, this is suggestive of adaptation of
558 expression following initial change caused by the ethanol. This gene codes for
559 the μ-opioid receptor (MOR) protein. With respect to drug preference, MOR-KO
560 mice (homozygous and heterozygous) show reduced self-administration of
561 ethanol (Hall et al., 2001). MORs are widely distributed around the mammalian
562 ventral and dorsal striatum (Arvidsson et al., 1995) and have been linked to the
563 appetitive phase of instrumental learning (Mucha and Herz, 1985). This suggests
564 that a reduction in MOR will reduce the motivational value of rewards, thus
565 rendering the animal less sensitive to Pavlovian appetitive cues or conditioned
566 reinforcers (Balleine and Dickinson, 1998). This is somewhat at odds with our
567 observations here that ethanol-exposed fish show a reduction in MOR mRNA
568 expression at 7dpf, but an increased preference for ethanol in the CPP test.
569 Theories linking MOR genetic variants to addiction are common (Contet et al.,
570 2004). Depletion of MOR during learning by naloxone causes an increase in
571 habit-based learning (Wassum et al., 2009) consistent with our findings here. As
572 such, despite this mechanism being fairly speculative at this stage, it certainly
573 warrants further investigation.

574 In addition to changes in DRD3, DAT and opioid receptor we observed that
575 the ethanol-exposed larvae had differences in nAChR mRNA expression.
576 Specifically, we found an initial increase in *chrnb3* expression after acute ethanol

exposure (50hpf), followed by a decrease in expression at 7dpf. We also saw suppression of both *chrna2* and *chrna3* mRNA expression following acute exposure, but no differences in expression in these genes later in development (i.e., at 7dpf). *chrnb3* encodes the β-3 nAChR subunit (nAChR-b3). Humans with specific allelic variants at this locus have previously been shown to show stronger preference for nicotine (Zeiger et al., 2008) and alcohol (Hoft et al., 2009). This latter study may explain why our fish showed a stronger preference for ethanol in the CPP test. However, as stated earlier, increased preference for drugs does not necessarily translate into, nor is it necessarily a predictor for, addiction. Interestingly, and of relevance to our observation that ethanol exposure increases habit-learning, variants at the nAChR-b3 locus have recently been shown to predict differences in addictive behaviour, i.e., as measured by the Fagerström Test for Cigarette Dependence (Rice et al., 2012a). This suggests that variation at this locus may predict behaviours that are related to dependence rather than simply heavy use of drugs (e.g., compulsive drug use). In mammals, nAChRb3s are located in a the form of hetero-oligomer populations (α6β2β3, α6α4β2β3), on DS dopamine neurons the region of the striatum responsible for the shift from cognitive to habit learning (Balleine and Dickinson, 1998). They are also located on VTA dopamine neurons (α6β2β3), a key component of the mesoaccumbens dopamine pathway, and on neurons in the habenula-interpeduncular (IPN) system (α3β3β4), thought to mediate aspects of withdrawal from drugs (Changeux, 2010; Grady et al., 2009). In zebrafish, the same nAChR subunits have been identified and serve a similar function as in mammals; they are located mainly pre-synaptically and mediate the release of other neurotransmitters (e.g., dopamine) (Ackerman et al., 2009; Zirger et al., 2003). Therefore, nAChR-b3 may

be important for the regulation of dopamine release in the striatum during learning (Azam et al., 2002; Cui et al., 2003; Zhou et al., 2001), thus helping to facilitate the process of habit learning.

Finally, we found that ethanol was related to changes in *chrna2* and *chrna3* mRNA expression following acute ethanol administration (50hpf). These changes were not observed later in development. *chrna2*, which encodes for the α2 nAChR subtype (nAChRa2) and *chrna3,* which encodes for the α3 nAChR (nAChRa3) subtype, are expressed in the zebrafish IPN and habenula, respectively, in a similar fashion to mammals (Hong et al., 2013). nAChRa2 in the IPN (Salas et al., 2009) and nAChRa3 in the habenula (Jackson et al., 2013) have been linked to the withdrawal from nicotine in rodent models, but not yet to withdrawal from other drugs of abuse, such as alcohol. However, both the IPN and the habenula receive major afferent inputs from the hypothalamus, limbic forebrain and mesoaccumbens pathway (Bianco and Wilson, 2009), and may regulate dopamine activity in reward pathways (Matsumoto and Hikosaka, 2007).

Finally, in this study we chose to use embryos at different developmental stages to examine gene expression. The reason for this was to exclude the possibility of social or environmental variables (which we did not vary systematically in this study) affecting gene expression. We accept that there is a possibility that phenotypes will alter as a result of gene-environment interactions during development, and the precise roles that this has to play in the development of different behavioural phenotypes (e.g., addiction) in later life would be an interesting area of future study.

In summary, we tested the hypotheses that exposure to moderate levels of ethanol during early brain ontogeny would result in enhanced place preference

for ethanol and increased habit formation during learning. **We found support for both of these hypotheses, indicating that in fish, exposure to ethanol developmentally has an impact on later behaviour and learning.** We also found that ethanol exposure during this critical developmental stage caused specific changes in dopamine and nicotinic receptor gene expression. **Collectively, these data suggest a possible molecular mechanism by which prenatal alcohol exposure may increase the risk of addiction in later life**. **The translational relevance of this in the fish is borne out in the structural and functional homology in brain regions related to these behavioural systems and neural pathways between the species (Guo, 2004; Parker et al., 2013a; Parker and Brennan, 2012), and existing evidence that humans exposed to moderate levels of ethanol prenatally are susceptible to addiction in later life (Baer et al., 2003).** Although speculative at present, it may be that ethanol exposure during early brain development causes subtle changes in the balance of nAChR in the IPN-habenular pathway that affects development of dopamine systems. This model will need to be examined in more detail in the future, for example by exposing both nAChR or DAT KO animals to ethanol and examining mRNA expression and behaviour during development and in adulthood.

References

Ackerman KM, Nakkula R, Zirger JM, Beattie CE, Boyd RT (2009) Cloning and spatiotemporal expression of zebrafish neuronal nicotinic acetylcholine receptor alpha 6 and alpha 4 subunit RNAs. Developmental Dynamics 238:980-992.

Alati R, Al Mamun A, Williams GM, O'Callaghan M, Najman JM, Bor W (2006) In utero alcohol exposure and prediction of alcohol disorders in early adulthood: a birth cohort study. Archives of general psychiatry 63:1009-1016.

Alati R, Macleod J, Hickman M, Sayal K, May M, Smith GD, Lawlor DA (2008) Intrauterine exposure to alcohol and tobacco use and childhood IQ: findings from a parental-offspring comparison within the Avon Longitudinal Study of Parents and Children. Pediatric research 64:659-666.

Arias C, Chotro MG (2005) Increased preference for ethanol in the infant rat after prenatal ethanol exposure, expressed on intake and taste reactivity tests. Alcoholism, clinical and experimental research 29:337-346.

Arvidsson U, Riedl M, Chakrabarti S, Lee J-H, Nakano AH, Dado R, Loh HH, Law P-Y, Wessendorf MW, Elde R (1995) Distribution and targeting of a mu-opioid receptor (MOR1) in brain and spinal cord. The Journal of neuroscience 15:3328-3341.

Azam L, Winzer‐Serhan UH, Chen Y, Leslie FM (2002) Expression of neuronal nicotinic acetylcholine receptor subunit mRNAs within midbrain dopamine neurons. Journal of Comparative Neurology 444:260-274.

Baer JS, Sampson PD, Barr HM, Connor PD, Streissguth AP (2003) A 21-year longitudinal analysis of the effects of prenatal alcohol exposure on young adult drinking. Archives of general psychiatry 60:377-385.

Balleine BW, Dickinson A (1998) Goal-directed instrumental action: contingency and incentive learning and their cortical substrates. Neuropharmacology 37:407-419.

Barbier E, Houchi H, Warnault V, Pierrefiche O, Daoust M, Naassila M (2009) Effects of prenatal and postnatal maternal ethanol on offspring response to alcohol and psychostimulants in long evans rats. Neuroscience 161:427-440.

Belin D, Belin-Rauscent A, Murray JE, Everitt BJ (2013) Addiction: failure of control over maladaptive incentive habits. Current opinion in neurobiology 23:564-572.

Belin D, Mar AC, Dalley JW, Robbins TW, Everitt BJ (2008) High impulsivity predicts the switch to compulsive cocaine-taking. Science 320:1352-1355.

Bianco IH, Wilson SW (2009) The habenular nuclei: a conserved asymmetric relay station in the vertebrate brain. Philosophical Transactions of the Royal Society B: Biological Sciences 364:1005-1020.

684 Blanchard BA, Steindorf S, Wang S, LeFevre R, Mankes RF, Glick SD (1993)
685 Prenatal ethanol exposure alters ethanol-induced dopamine release in nucleus
686 accumbens and striatum in male and female rats. Alcoholism, clinical and
687 experimental research 17:974-981.

688 Brembs B (2009) Mushroom Bodies Regulate Habit Formation in *Drosophila*.
689 Current Biology 19:1351-1355.

690 Brennan CH, Parmar A, Kily LKM, Ananthathevan A, Doshi A, Patel S, Kalueff A
691 (2011) Conditioned Place Preference Models of Drug Dependence and Relapse
692 to Drug Seeking: Studies with Nicotine and Ethanol. Neuromethods 52:163-180.

693 Brus R, Felinska W, Rykaczewska M, Kostrzewa RM, Szkilnik R, Plech A (1995)
694 Prenatal ethanol diminishes reactivity of presumed dopamine D3 receptors in
695 rats. Polish journal of pharmacology 47:109-114.

696 Changeux J-P (2010) Nicotine addiction and nicotinic receptors: lessons from
697 genetically modified mice. Nature Reviews Neuroscience 11:389-401.

698 Choong K, Shen R (2004) Prenatal ethanol exposure alters the postnatal
699 development of the spontaneous electrical activity of dopamine neurons in the
700 ventral tegmental area. Neuroscience 126:1083-1091.

701 Contet C, Kieffer BL, Befort K (2004) Mu opioid receptor: a gateway to drug
702 addiction. Current opinion in neurobiology 14:370-378.

703 Corbit LH, Muir JL, Balleine BW (2001) The role of the nucleus accumbens in
704 instrumental conditioning: evidence of a functional dissociation between
705 accumbens core and shell. The Journal of Neuroscience 21:3251-3260.

706 Cui C, Booker T, Allen RS, Grady SR, Whiteaker P, Marks MJ, Salminen O, Tritto
707 T, Butt CM, Allen W (2003) The β3 nicotinic receptor subunit: a component of α-
708 conotoxin MII-binding nicotinic acetylcholine receptors that modulate dopamine
709 release and related behaviors. The Journal of neuroscience 23:11045-11053.

710 Dalley JW, Fryer TD, Brichard L, Robinson ES, Theobald DE, Laane K, Pena Y,
711 Murphy ER, Shah Y, Probst K, Abakumova I, Aigbirhio FI, Richards HK, Hong Y,
712 Baron JC, Everitt BJ, Robbins TW (2007) Nucleus accumbens D2/3 receptors
713 predict trait impulsivity and cocaine reinforcement. Science 315:1267-1270.

714 Darland T, Dowling JE (2001) Behavioral screening for cocaine sensitivity in
715 mutagenized zebrafish. Proceedings of the National Academy of Sciences of the
716 United States of America 98:11691-11696.

717 Dlugos CA, Rabin RA (2003) Ethanol effects on three strains of zebrafish: model
718 system for genetic investigations. Pharmacology, biochemistry, and behavior
719 74:471-480.

720 Dominguez HD, Chotro MG, Molina JC (1993) Alcohol in the amniotic fluid prior to
721 cesarean delivery: effects of subsequent exposure to the drug's odor upon
722 alcohol responsiveness. Behavioral and neural biology 60:129-138.

Everitt BJ, Belin D, Economidou D, Pelloux Y, Dalley JW, Robbins TW (2008) Review. Neural mechanisms underlying the vulnerability to develop compulsive drug-seeking habits and addiction. Philosophical transactions of the Royal Society of London Series B, Biological sciences 363:3125-3135.

Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nature neuroscience 8:1481-1489.

Everitt BJ, Robbins TW (2013) From the ventral to the dorsal striatum: devolving views of their roles in drug addiction. Neuroscience & Biobehavioral Reviews 37:1946-1954.

Faas AE, Sponton ED, Moya PR, Molina JC (2000) Differential responsiveness to alcohol odor in human neonates: effects of maternal consumption during gestation. Alcohol 22:7-17.

Fernandez-Ruiz J, Wang J, Aigner TG, Mishkin M (2001) Visual habit formation in monkeys with neurotoxic lesions of the ventrocaudal neostriatum. Proceedings of the National Academy of Sciences 98:4196-4201.

Franco R, Ferré S, Agnati L, Torvinen M, Ginés S, Hillion J, Casadó V, Lledó P-M, Zoli M, Lluis C (2000) Evidence for adenosine/dopamine receptor interactions: indications for heteromerization. Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 23:S50-S59.

Fulginiti S, Minetti SA, Virgolini MB (1994) Effects of acute ethanol intoxication during pregnancy on central dopaminergic system in male rats. Neurotoxicology and teratology 16:385-389.

Gemenetzidis E, Elena-Costea D, Parkinson EK, Waseem A, Wan H, Teh M-T (2010) Induction of human epithelial stem/progenitor expansion by FOXM1. Cancer research 70:9515-9526.

Grady SR, Moretti M, Zoli M, Marks MJ, Zanardi A, Pucci L, Clementi F, Gotti C (2009) Rodent Habenulo–Interpeduncular Pathway Expresses a Large Variety of Uncommon nAChR Subtypes, But Only the α3β4 and α3β3β4 Subtypes Mediate Acetylcholine Release. The Journal of Neuroscience 29:2272-2282.

Guo S (2004) Linking genes to brain, behavior and neurological diseases: what can we learn from zebrafish? Genes, brain, and behavior 3:63-74.

Guo S, Brush J, Teraoka H, Goddard A, Wilson SW, Mullins MC, Rosenthal A (1999) Development of noradrenergic neurons in the zebrafish hindbrain requires BMP, FGF8, and the homeodomain protein soulless/Phox2a. Neuron 24:555-566.

Hall F, Sora I, Uhl G (2001) Ethanol consumption and reward are decreased in μ-opiate receptor knockout mice. Psychopharmacology 154:43-49.

Hanson JW, Jones KL, Smith DW (1976) Fetal alcohol syndrome. JAMA: the journal of the American Medical Association 235:1458.

762 Hironaka N, Ikeda K, Sora I, Uhl GR, Niki H (2004) Food‐Reinforced Operant
763 Behavior in Dopamine Transporter Knockout Mice: Enhanced Resistance to
764 Extinction. Annals of the New York Academy of Sciences 1025:140-145.

765 Hoft NR, Corley RP, McQueen MB, Huizinga D, Menard S, Ehringer MA (2009)
766 SNPs in CHRNA6 and CHRNB3 are associated with alcohol consumption in a
767 nationally representative sample. Genes, Brain and Behavior 8:631-637.

768 Hong E, Santhakumar K, Akitake CA, Ahn SJ, Thisse C, Thisse B, Wyart C,
769 Mangin J-M, Halpern ME (2013) Cholinergic left-right asymmetry in the habenulo-
770 interpeduncular pathway. Proceedings of the National Academy of Sciences
771 110:21171-21176.

772 Jackson KJ, Sanjakdar SS, Muldoon PP, McIntosh JM, Damaj MI (2013) The
773 α3β4* nicotinic acetylcholine receptor subtype mediates nicotine reward and
774 physical nicotine withdrawal signs independently of the α5 subunit in the mouse.
775 Neuropharmacology 70:228-235.

776 Kalueff AV, Gebhardt M, Stewart AM, Cachat JM, Brimmer M, Chawla JS,
777 Craddock C, Kyzar EJ, Roth A, Landsman S (2013) Towards a comprehensive
778 catalog of zebrafish behavior 1.0 and beyond. Zebrafish 10:70-86.

779 Keil V, Paley B, Frankel F, O'Connor MJ (2010) Impact of a social skills
780 intervention on the hostile attributions of children with prenatal alcohol exposure.
781 Alcoholism, clinical and experimental research 34:231-241.

782 Kelly SJ, Day N, Streissguth AP (2000) Effects of prenatal alcohol exposure on
783 social behavior in humans and other species. Neurotoxicology and teratology
784 22:143-149.

785 Kéri S, Juhász A, Rimanóczy Á, Szekeres G, Kelemen O, Cimmer C, Szendi I,
786 Benedek G, Janka Z (2005) Habit Learning and the Genetics of the Dopamine
787 D□ Receptor: Evidence From Patients With Schizophrenia and Healthy Controls.
788 Behavioral neuroscience 119:687.

789 Kily LJM, Cowe YCM, Hussain O, Patel S, McElwaine S, Cotter FE, Brennan CH
790 (2008) Gene expression changes in a zebrafish model of drug dependency
791 suggest conservation of neuro-adaptation pathways. Journal of Experimental
792 Biology 211:1623-1634.

793 Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of
794 embryonic development of the zebrafish. Developmental dynamics : an official
795 publication of the American Association of Anatomists 203:253-310.

796 Kitabatake Y, Hikida T, Watanabe D, Pastan I, Nakanishi S (2003) Impairment of
797 reward-related learning by cholinergic cell ablation in the striatum. Proceedings of
798 the National Academy of Sciences 100:7965-7970.

799 Landgren M, Svensson L, Strömland K, Grönlund MA (2010) Prenatal alcohol
800 exposure and neurodevelopmental disorders in children adopted from Eastern
801 Europe. Pediatrics 125:e1178-e1185.

Malanga CJ, 3rd, Kosofsky BE (1999) Mechanisms of action of drugs of abuse on the developing fetal brain. Clinics in perinatology 26:17-37, v-vi.

Mathur P, Berberoglu MA, Guo S (2011) Preference for ethanol in zebrafish following a single exposure. Behavioural brain research 217:128-133.

Matsumoto M, Hikosaka O (2007) Lateral habenula as a source of negative reward signals in dopamine neurons. Nature 447:1111-1115.

May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE (2009) Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. Developmental Disabilities Research Reviews 15:176-192.

Middaugh LD, Boggan WO, Shepherd CL (1994) Prenatal ethanol effects and dopamine systems of adult C57 male mice. Neurotoxicology and teratology 16:207-212.

Miller MW (1999) A longitudinal study of the effects of prenatal ethanol exposure on neuronal acquisition and death in the principal sensory nucleus of the trigeminal nerve: interaction with changes induced by transection of the infraorbital nerve. Journal of neurocytology 28:999-1015.

Miura M, Masuda M, Aosaki T (2008) Roles of μ-opioid receptors in GABAergic synaptic transmission in the striosome and matrix compartments of the striatum. Molecular neurobiology 37:104-115.

Moore KA, Kunsman GW, Levine BS, Herman MM, Cervenak J, Hyde TM (1997) A comparison of ethanol concentrations in the occipital lobe and cerebellum. Forensic science international 86:127-134.

Mucha RF, Herz A (1985) Motivational properties of kappa and mu opioid receptor agonists studied with place and taste preference conditioning. Psychopharmacology 86:274-280.

Ninkovic J, Folchert A, Makhankov YV, Neuhauss SCF, Sillaber I, Straehle U, Bally‐Cuif L (2006) Genetic identification of AChE as a positive modulator of addiction to the psychostimulant D‐amphetamine in zebrafish. Journal of neurobiology 66:463-475.

Olson HC, Feldman JJ, Streissguth AP, Sampson PD, Bookstein FL (1998) Neuropsychological deficits in adolescents with fetal alcohol syndrome: clinical findings. Alcoholism, clinical and experimental research 22:1998-2012.

Packard MG, Knowlton BJ (2002) Learning and memory functions of the basal ganglia. Annual review of neuroscience 25:563-593.

Paintner A, Williams AD, Burd L (2012) Fetal alcohol spectrum disorders--implications for child neurology, part 1: prenatal exposure and dosimetry. Journal of child neurology 27:258-263.

Parker M, Brock A, Walton R, Brennan C (2013a) The role of zebrafish (Danio rerio) in dissecting the genetics and neural circuits of executive function. Frontiers in neural circuits 7:63.

Parker M, McBride SD, Redhead E, Goodwin D (2009) Differential place and response learning in horses displaying an oral stereotypy. Behavioural Brain Research 200:100-105.

Parker MO, Annan LV, Kanellopoulos AH, Brock AJ, Combe FJ, Baiamonte M, Teh M-T, Brennan CH (In Press) The utility of zebrafish to study the mechanisms by which ethanol affects social behavior and anxiety during early brain development. Progress in Neuro-Psychopharmacology and Biological Psychiatry.

Parker MO, Brennan CH (2012) Zebrafish (Danio rerio) models of substance abuse: Harnessing the capabilities. Behaviour 149:1037 – 1062

Parker MO, Brock AJ, Millington ME, Brennan CH (2013b) Behavioral Phenotyping of Casper Mutant and 1-Pheny-2-Thiourea Treated Adult Zebrafish. Zebrafish 10:466-471.

Parker MO, Brock AJ, Sudwarts A, Brennan CH (2014) Atomoxetine reduces anticipatory responding in a 5-choice serial reaction time task for adult zebrafish. Psychopharmacology:1-9.

Parker MO, Ife D, Ma J, Pancholi M, Smeraldi F, Straw C, Brennan CH (2013c) Development and automation of a test of impulse control in zebrafish. Frontiers in systems neuroscience 7:63.

Reimers MJ, Flockton AR, Tanguay RL (2004) Ethanol-and acetaldehyde-mediated developmental toxicity in zebrafish. Neurotoxicology and teratology 26:769-781.

Rice JP, Hartz SM, Agrawal A, Almasy L, Bennett S, Breslau N, Bucholz KK, Doheny KF, Edenberg HJ, Goate AM (2012a) CHRNB3 is more strongly associated with Fagerström Test for Cigarette Dependence - based nicotine dependence than cigarettes per day: phenotype definition changes genome - wide association studies results. Addiction 107:2019-2028.

Rice JP, Suggs LE, Lusk AV, Parker MO, Candelaria-Cook FT, Akers KG, Savage DD, Hamilton DA (2012b) Effects of exposure to moderate levels of ethanol during prenatal brain development on dendritic length, branching, and spine density in the nucleus accumbens and dorsal striatum of adult rats. Alcohol 46:577-584.

Robbins TW, Everitt BJ (1999) Drug addiction: bad habits add up. Nature 398:567-570.

Salas R, Sturm R, Boulter J, De Biasi M (2009) Nicotinic receptors in the habenulo-interpeduncular system are necessary for nicotine withdrawal in mice. The Journal of Neuroscience 29:3014-3018.

879 Satterthwaite F (1946) OF VARIANCE COMPONENTS. Biometrics 2:110-114.

880 Sood B, Delaney-Black V, Covington C, Nordstrom-Klee B, Ager J, Templin T,
881 Janisse J, Martier S, Sokol RJ (2001) Prenatal alcohol exposure and childhood
882 behavior at age 6 to 7 years: I. dose-response effect. Pediatrics 108:E34.

883 Spear NE, Molina JC (2005) Fetal or infantile exposure to ethanol promotes
884 ethanol ingestion in adolescence and adulthood: a theoretical review. Alcoholism,
885 clinical and experimental research 29:909-929.

886 Streissguth AP, Bookstein FL, Sampson PD, Barr HM (1989) Neurobehavioral
887 effects of prenatal alcohol: Part III. PLS analyses of neuropsychologic tests.
888 Neurotoxicology and teratology 11:493-507.

889 Streissguth AP, Sampson PD, Olson HC, Bookstein FL, Barr HM, Scott M,
890 Feldman J, Mirsky AF (1994) Maternal drinking during pregnancy: attention and
891 short-term memory in 14-year-old offspring--a longitudinal prospective study.
892 Alcoholism, clinical and experimental research 18:202-218.

893 Suess PE, Newlin DB, Porges SW (1997) Motivation, sustained attention, and
894 autonomic regulation in school-age boys exposed in utero to opiates and alcohol.
895 Experimental and clinical psychopharmacology 5:375-387.

896 Tang R, Dodd A, Lai D, McNabb WC, Love DR (2007) Validation of zebrafish
897 (Danio rerio) reference genes for quantitative real-time RT-PCR normalization.
898 Acta biochimica et biophysica Sinica 39:384-390.

899 Teh MT, Hutchison IL, Costea DE, Neppelberg E, Liavaag PG, Purdie K,
900 Harwood C, Wan H, Odell EW, Hackshaw A (2013) Exploiting FOXM1 -
901 orchestrated molecular network for early squamous cell carcinoma diagnosis and
902 prognosis. International Journal of Cancer 132:2095-2106.

903 Thomas SE, Kelly SJ, Mattson SN, Riley EP (1998) Comparison of social abilities
904 of children with fetal alcohol syndrome to those of children with similar IQ scores
905 and normal controls. Alcoholism: Clinical and Experimental Research 22:528-533.

906 Valenzuela CF, Morton RA, Diaz MR, Topper L (2012) Does moderate drinking
907 harm the fetal
908 brain? Insights from animal models. Trends in Neurosciences 35:284 - 292.

909 Van Waes V, Enache M, Berton O, Vinner E, Lhermitte M, Maccari S, Darnaudery
910 M (2011) Effect of prenatal stress on alcohol preference and sensitivity to chronic
911 alcohol exposure in male rats. Psychopharmacology 214:197-208.

912 van Wieringen H, Letteboer TG, Pereira RR, de Ruiter S, Balemans WA, Lindhout
913 D (2010) [Diagnosis of fetal alcohol spectrum disorders]. Nederlands tijdschrift
914 voor geneeskunde 154:A331.

915 Wang J, Haj-Dahmane S, Shen RY (2006) Effects of prenatal ethanol exposure
916 on the excitability of ventral tegmental area dopamine neurons in vitro. The
917 Journal of pharmacology and experimental therapeutics 319:857-863.

Wassum K, Cely I, Maidment N, Balleine B (2009) Disruption of endogenous opioid activity during instrumental learning enhances habit acquisition. Neuroscience 163:770-780.

Westerfield M (1993) *The zebrafish book: a guide for the laboratory use of zebrafish (Brachydanio rerio)*. M. Westerfield Eugene, OR.

Xu F, Gainetdinov RR, Wetsel WC, Jones SR, Bohn LM, Miller GW, Wang Y-M, Caron MG (2000) Mice lacking the norepinephrine transporter are supersensitive to psychostimulants. Nature neuroscience 3:465-471.

Yin HH, Knowlton BJ (2004) Contributions of striatal subregions to place and response learning. Learning & Memory 11:459-463.

Yin HH, Knowlton BJ, Balleine BW (2004) Lesions of dorsolateral striatum preserve outcome expectancy but disrupt habit formation in instrumental learning. European Journal of Neuroscience 19:181-189.

Yin HH, Ostlund SB, Balleine BW (2008) Reward‐guided learning beyond dopamine in the nucleus accumbens: the integrative functions of cortico‐basal ganglia networks. European Journal of Neuroscience 28:1437-1448.

Yu C, Gupta J, Chen J-F, Yin HH (2009) Genetic deletion of A2A adenosine receptors in the striatum selectively impairs habit formation. The Journal of Neuroscience 29:15100-15103.

Zeiger JS, Haberstick BC, Schlaepfer I, Collins AC, Corley RP, Crowley TJ, Hewitt JK, Hopfer CJ, Lessem J, McQueen MB (2008) The neuronal nicotinic receptor subunit genes (CHRNA6 and CHRNB3) are associated with subjective responses to tobacco. Human molecular genetics 17:724-734.

Zhou F-M, Liang Y, Dani JA (2001) Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. Nature neuroscience 4:1224-1229.

Zirger JM, Beattie CE, McKay DB, Thomas Boyd R (2003) Cloning and expression of zebrafish neuronal nicotinic acetylcholine receptors. Gene expression patterns 3:747-754.

Figure 1. Time spent in vicinity of conditioned cue after 2 conditioning sessions in CPP task for fish developmentally exposed to 20mM ethanol and handling controls. ***p* = 0.01.

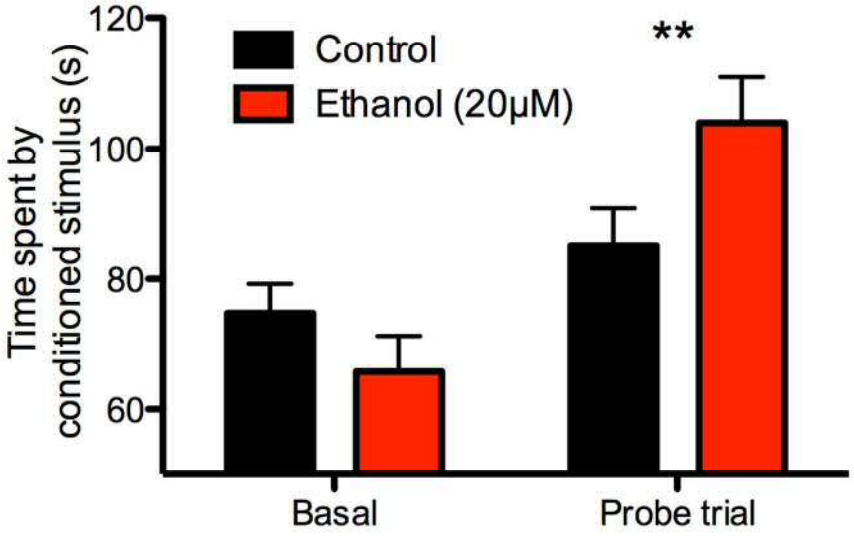
Figure 2. Acquisition graphs for training on T-maze. Left hand panel displays the proportion of correct trials in each session (A); Right hand panel displays the approach latency (s) (B);. Learning criterion was set at both groups showing mean approach latencies of < 20 sec for 5-consecutive trials and both groups >75% correct.

Figure 3. Changes in mRNA expression of nAChRs *chrna2 (*A*), chrna3* (B)*, chrnb3* (C)*,* dopamine transporter (D) and dopamine D3 receptor, *drd3* (E)*,* and μ-opioid receptor, *oprm1* (F), normalised against *β-actin*, *ef1α* and *rpl13α*, following exposure to moderate levels of ethanol during early brain development.

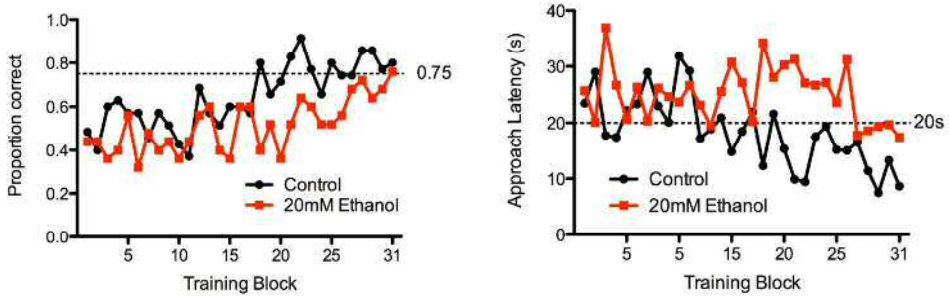
Supplementary Figure 1. Arena used for CPP assay. Basal preference was ascertained, and the fish was conditioned to the least preferred side. Drug was added to both sides of the apparatus to ensure equal dispersion across the tank.

Supplementary Figure 2. T-maze used to test fish in a place-response test. Fish were initially entered into the south (S) arm (nb, this was counterbalanced between fish, so half started in the north (N) arm). The barrier was released and the fish was reinforced for entries to the east (E) arm (nb, this was counterbalanced, so half were reinforced in the west (W) arm). During the probe trial, the fish was released from the opposite arm to that from which it was released during training, and the direction of its response was noted.

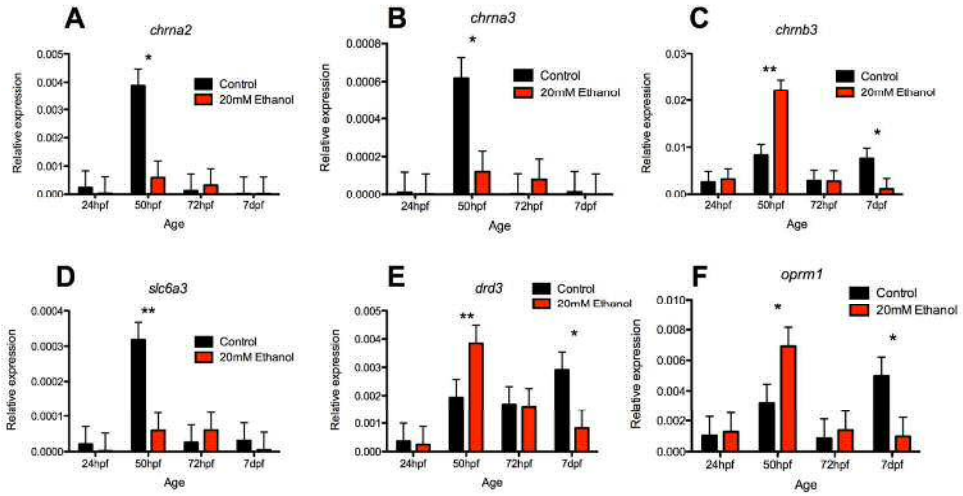
Table 1. *Primer Sequences for Quantitative Real-Time PCR Assessment of mRNA Expression*



111x70mm (300 x 300 DPI)



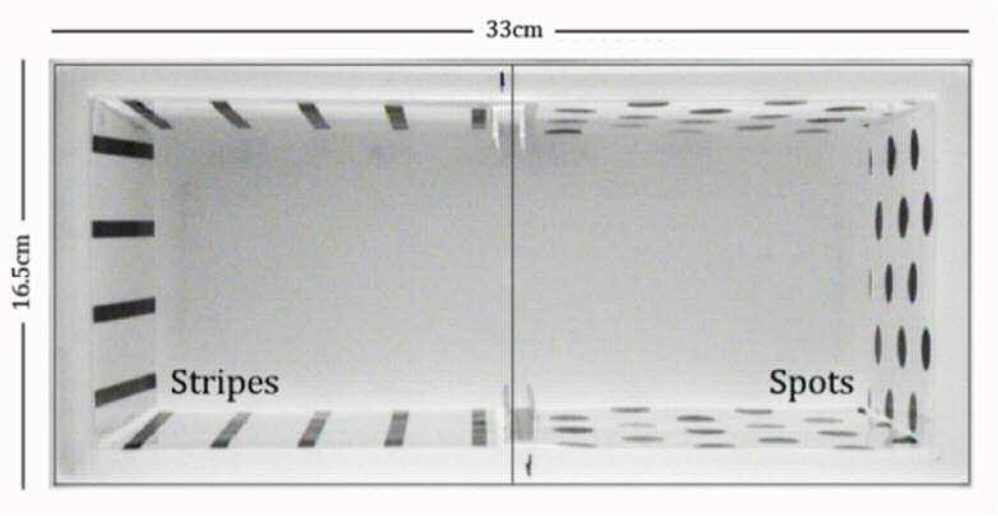
249x90mm (300 x 300 DPI)



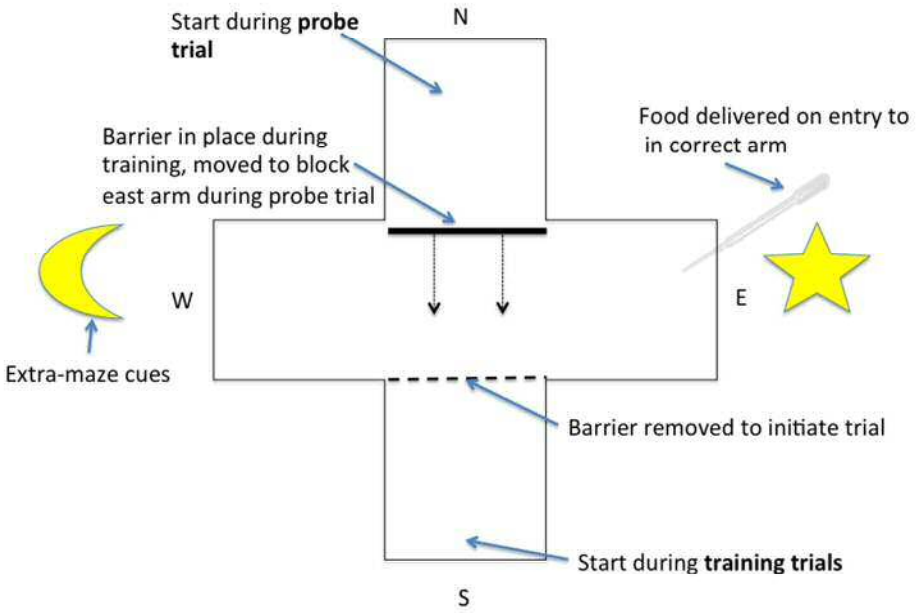
248x132mm (300 x 300 DPI)

Gene name	Primers
adora2aa-F	CTT GAG CGC AGG AAC CAG AG
adora2aa-R	CGC GCA CTG AGA GAT GAC AG
adora2ab-F	CTG GTG ATT GCG GTT TTG GC
adora2ab-R	CAG GAC TCC GAC TGC AAT GT
β-actin-F	CGA GCT GTC TTC CCA TCC A
β-actin-R	TCA CCA ACG TAG CTG TCT TTC TG
rpl13α-F	TCT GGA GGA CTG TAA GAG GTA TGC
rpl13α-R	AGA CGC ACA ATC TTG AGA GCA G
eF1α-F	CTG GAG GCC AGC TCA AAC AT
eF1α-R	ATC AAG AAG AGT AGT ACC GCT AGC ATT AC
chrna2-F	GCG GAA AAC CGG ATA AAA ACA CTC
chrna2-R	AGT TTG TCC TCT GCG TGT GCA T
chrna3-F	TGT ACA TCC GCC GAT TAC CGC T
chrna3-R	TCC GCA GTC GGA GGG CAG TA
chrna4-F	TTA CAA GAG GTT TGG GCG CT
chrna4-R	ACA GAC CAG TAG ATC ATC ACT CC
chrna5-F	GGC TCC CAG GTC GAC ATT
chrna5-R	AAC CCC GGT TAC CAG TGG CCT
chrna6-F	CTT TGG GCC TCT TCC TGC AA
chrna6-R	TCA GAG TCT TGA TGT AGT GAC GG
chrna7-F	ACC GTG TCA CAT TGT TCA TTC TC
chrna7-R	ACA GGT CTC TCC AGT GGG TTA
chrnb2-F	GGC TGC CTG ATG TTG TTC TT

chrnb2-R	TGG TGG CAA CCA GAA GAC ACT T
chrnb3-F	CAG GAG TCA ACC TCC GCT TT
chrnb3-R	TGA ATC TGA ACG CAC TGG CT
chrnb4-F	TGA TCA CAT GAT GGG GAA TGA CG
chrnb4-R	CAC CAC ACA CAC GAT CAC AAA G
drd1-F	TGG TTC CTT TCT GCA ACC CA
drd1-R	AGT GAT GAG TTC GCC CAA CC
drd2-F	TCC ACA AAA TCA GGA AAA GCG T
drd2-R	CAG CCA ATG TAA ACC GGC AA
drd3-F	ATC GAG TTT CGC AGA GCC TT
drd3-R	TCC ACA GTG TCT GAA AGC CG
oprm1-F	CCG TAT GTG ACA GGA CGC CA
oprm1-R	TTT CCC ACC AGT CCC ATC ACA
slc6a2-F	AGG TGA CAT TGT TTG AGA TGT CTT
slc6a2-R	TGT CTT GGT AGT GTC AAG TTG T
slc6a3-F	TAT GTG GTC CTG ACC GTG CT
slc6a3-R	CAC ATG TGT AGG CGC AGG AA



254x142mm (72 x 72 DPI)



352x264mm (72 x 72 DPI)